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Research Article

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Response surface optimization of medium composition for alkaline protease production by *Bacillus cereus* strain S8

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ABSTRACT

A culture medium for production of bacterial alkaline protease was developed following preliminary studies by means of response surface method. Central composite design was applied to optimize the medium constituents and explain the combined effects of three medium constituents, viz., molasses, potassium nitrate and inoculum size. The optimum values for the tested variables were molasses (1%), potassium nitrate (0.75%) and inoculum size (1%). A second-order model equation was suggested and then validated experimentally. The model adequacy was very satisfactory. The B. cereus strain S8 produced 205±0.35U/ml protease under experimental optimized culture conditions with these three significant variables over the basal medium (165±0.28 U/ml).

Key words: *Alkaline protease; Bacillus cereus strain S8; Medium optimization; Response surface methodology, central composite design.*

INTRODUCTION

Proteases represent the class of enzymes which occupy a pivotal position with respect to their physiological roles as well as their commercial applications in different industries *viz.*, detergent, food, pharmaceutical, leather and for recovery of silver from used x-ray films etc. Microbial proteases represent one of the three largest groups of industrial enzymes and account for approximately 60% of the total enzyme sale in the world[1]. Microbial proteases are classified as acidic, neutral and alkaline depending on the pH at which they show maximum activity. Amongst these, alkaline proteases find a wide range of applications in laundry detergent, textile, food processing, pharmaceuticals, leather, paper and pulp industries[1–3].

Alkaline proteases are produced by a wide range of microorganisms including bacteria, molds, yeasts and also mammalian tissues. Currently, a large proportion of commercially available alkaline proteases are derived from *Bacillus* strains[3]. Media components were found to have great influence on extracellular protease production and are different for each microorganism. Therefore, the required constituents and their concentrations have to be optimized accordingly[4–9]. Industrial fermentation is moving away from traditional and largely empirical operation towards knowledge based and better controlled process[10]. A number of optimization techniques could be used for this purpose. Statistical approaches offer ideal ways for process optimization studies in biotechnology[9,11,12]. Time consuming, requirement of more experimental data sets[9,13] and missing the interactions among parameters are the obstacles in predicting the accurate results when the conventional optimization procedures like 'one-factor at a time' were applied[14,15]. On the contrary, statistical procedures have advantages basically due to utilization of fundamental principles of statistics, randomization, replication and duplication[16]. Response surface method (RSM) is one of the popularly used optimization procedures, mainly developed based on full

factorial central composite design (CCD)[17]. Response surface method (RSM) is a collection of mathematical and statistical techniques that are useful for modeling and analysis in applications where a response of interest is influenced by several variables and the objective is to optimize this response[18]. Response surface method (RSM) helps identify the effective factors, study interactions, select optimum conditions and quantify the relationships between one or more measured responses and the vital input factors in limited number of experiments4,[14,15]. Several fermentation processes have been optimized using this methodology[19]. A CCD has three groups of design points: two-level factorial or fractional factorial, axial and central points. Central composite design is designed to estimate the coefficients of a quadratic model[18]. Several reports on the central composite design are available in the literature[5,15,20]. In this study, protease production from *Bacillus cereus* strain S8 as a result of the interaction between three variables, molasses, inoculum size and KNO_3, which had played a significant role in enhancing the production of alkaline protease, was optimized with response surface methodology.

MATERIALS AND METHODS

Microorganism and culture maintenance

The microorganism used in this study was isolated from soil samples[21] screened using a skim milk agar plate depending up on the zone of hydrolysis. It was identified as *Bacillus cereus* strain S8 (MTCC No: 11901) according to morphological, biochemical tests and 16S r RNA gene sequencing[22].

Stock cultures of the isolate were stored in 30% glycerol at -70^0C. Protease enzyme production was carried out using standard media glucose, 0.5% (w/v); peptone, 0.75% (w/v); salt solution, 5% (v/v) - {($MgSo_4.7H_2O$, 0.5% (w/v); KH_2PO_4 0.5% (w/v)}; and $FeSO_4.7H_2O$, 0.01% (w/v) at 160rpm.This basal medium was used for the preliminary studies of the bacterial growth and protease production.

Inoculum preparation

For enzyme production, bacterial cells from a 24 h aged culture were inoculated into 250 ml Erlenmeyer flasks containing 50 ml of sterile inoculation medium. The composition of the inoculum medium was the same as basal medium. The cultures were grown at 37^0C for 24 h in a shaker incubator. After reaching the optimal growth, the culture was used to inoculate production flasks.

Growth conditions

The alkaline medium (pH 12.0) used for protease production contained: molasses (variable); inoculum size (variable); KNO_3 (variable); salt solution, 5% (v/v) - {($MgSo_4.7H_2O$, 0.5% (w/v); KH_2PO_4 0.5% (w/v)}; and $FeSO_4.7H_2O$, 0.01% (w/v). The operating conditions were maintained at a temperature of 37^0C for 72 h in a shaker incubator at 160rpm.

Protease assay

The enzyme activity was determined by using Mc Donald & Chen method[23].

Experimental design and optimization

In order to characterize how the significant factors affect the responses, it was attempted to improve the composition of the medium by comparing different levels of several factors that were found to have more influence on the production of protease by the bacterium *Bacillus cereus* strain S8. Based on the results obtained in preliminary experiments, molasses (%) - X1, nitrogen source KNO_3 (%) – X2 and inoculum size (%) – X_3, were found to be three major independent variables in the protease production. Response surface methodology (RSM) was used to estimate the main effects on response, i.e. protease yield (Y).

Variables evaluated include

X1 =% V/V molasses concentration

X2 = % w/w Potassium nitrate (KNO_3)

X3 = % V/V Inoculum size

The response variables include

Y1 = Protease activity

Central composite design (CCD) was used to find the optimal concentrations of these three factors. These independent variables were capable of influencing the alkaline protease production (Y) by *Bacillus cereus* strain S8 at pH 12.0 and 37^0C. These three factors with each coded levels consisting of 17 experimental runs were used to analyze the experimental data, to allow better estimate of the experimental error and to provide extra information about yields in the interior of the experimental region. The minimum and maximum range of variables investigated and the full experimental plan with respect to their coded values are listed in Tables 1 and 2. Response surface plots and statistical analyses were performed using Design expert Software ® (Minneapolis, MN 55413-2726, USA). All experiments were carried out in triplicates.

A multiple regression analysis of the data was carried out with the statistical package (Stat-Ease Inc., Minneapolis, MN, USA) and the second-order polynomial equation that defines predicted response (Y) in terms of the independent variables (X1, X2, and X3) was obtained:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_{21} + b_{22}X_{22} + b_{33}X_{23} + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3$$

Where b_0 is intercept term, b_1, b_2, b_3 linear coefficients, b_{11}, b_{22}, b_{33} squared coefficients and b_{12}, b_{23}, b_{13} are interaction coefficients. Combinations of factors (such as X_1X_2) represent an interaction between the individual factors in that term. Then the response is a function of the levels of factors. The response surface graphs indicate the effect of variables individually and in combination and determine their optimum levels for maximal protease production. To validate these predictions, flask cultivation using the completely optimized medium composition was carried out thrice.

RESULTS AND DISCUSSION

The central composite design was used to find the suitable concentrations of the variables on alkaline protease production by *Bacillus cereus* strain S8. The results of CCD experiments consisted of predicted and experimental data for studying the effects of three independent variables; *viz*., molasses, potassium nitrate and inoculum size on protease production are presented in Table 2. The data were fitted with a second-order polynomial function. The analysis of variance (Table 3) indicated that the model terms of *X*1, *X*2 and *X*3 were significant ("probe>*F*" less than 0.05).

The model F-value is 56.02. High F-value implies that the model is significant. The regression equation obtained from analysis of variance (ANOVA) indicated that the multiple correlation coefficient indicate the model can explain variation in the response. It should be noted that a R2 value greater than 0.75 indicates the aptness of the model. The adjusted R2 and predicted R2 values are 0.9687 and 0.7809 respectively. Also, the model has an "adequate precision value" of 19.682; this suggests that the model can be used to navigate the design space (Table 4). The "adequate precision value" is an index of the signal to noise ratio and a value >4 is an essential prerequisite for a model to be a good fit. The ANOVA results confirmed a satisfactory adjustment of the simplified quadratic model to the experimental data. The parity plot showed a satisfactory correlation between the experimental and predictive values (Fig. 1), wherein, the points clustered around the diagonal line which indicates the good fit of the model. The contour plot (Fig. 2a), three-dimensional response surface graphs (Fig. 2b) and overlay plots (data not shown) were plotted to show the interaction of the medium composition and the optimum concentrations of determined components on protease production. The maximum protease activity of 205.2U/ml was predicted by the model. The suggested medium composition was repeated thrice. The validation experiment showed that the experimentally determined production values were in close agreement with the statistically predicted ones, confirming the model's authenticity. The *B. cereus* strain S8 produced 205±0.35U/ml protease under experimental optimized culture conditions with these three significant variables than basal medium (165±0.28 U/ml). The desirability of the model is 1.0 (Fig.3), indicates the significance of the formulated medium by using this method.

Table 1: Experimental range and levels of the independent variables in media formulations

Variables	Range and levels		
	-1	0	+1
Molasses (%) X1	0.5	1.0	1.5
Nitrogen source (KNO_3) (%) X2	0.25	0.5	0.75
Inoculum size (%) X3	0.25	0.5	0.75

Table 2: Experimental design along with observed and predicted protease activity

S. No	Factor 1 (X_1) A. Molasses (%)	Factor 2 (X_2) A. Nitrogen source (%)	Factor (X_3) A. Inoculum size (%)	Response (Y) Protease activity(U/ml) Observed	Response (Y) Protease activity(U/ml) Predicted
1	1	0.5	1.5	183.4±0.11	184
2	1	0.75	1	163.1±0.27	166
3	1	0.25	2	151.4±0.19	151
4	1	0.5	1.5	183±0.32	184
5	1	0.75	2	205±0.35	205.2
6	0.5	0.25	1.5	90.7±0.39	91
7	0.5	0.5	1	110±0.15	110
8	1	0.25	1	163±0.17	164
9	0.5	0.75	1.5	72±0.26	72
10	1	0.5	1.5	183.4±0.22	184
11	1.5	0.75	1.5	114±0.47	115
12	0.5	0.5	2	90±0.18	90
13	1	0.5	1.5	183.4±0.24	184
14	1.5	0.5	1	79±0.10	81
15	1.5	0.25	1.5	60±0.21	61
16	1.5	0.5	2	140±0.09	142
17	1	0.5	1.5	183.4±0.14	184

Table 3: Analysis of Variance (ANOVA)

ANOVA for Response Surface Quadratic model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	31727.26	9	3525.25	56.02	< 0.0001	significant
A-Molasses	144.50	1	144.50	2.30	0.1735	Significant
B-Potassium nitrate	338.00	1	338.00	5.37	0.0536	Significant
C-Inoculum size	112.50	1	112.50	1.79	0.2230	Significant
AB	1332.25	1	1332.25	21.17	0.0025	Significant
AC	1560.25	1	1560.25	24.79	0.0016	significant
BC	42.25	1	42.25	0.67	0.4396	Significant
A^2	25207.96	1	25207.96	400.58	< 0.0001	Significant
B^2	2014.80	1	2014.80	32.02	0.0008	Significant
C^2	0.59	1	0.59	9.409E-003	0.9254	Significant
Residual	440.50	7	62.93			
Lack of Fit	440.50	3	146.83			
Pure Error	0.000	4	0.000			
Cor Total	32167.76	16				

Table 4: Regression analysis

Std. Dev.	7.93	**R-Squared**	0.9863
Mean	137.12	**Adj R-Squared**	0.9687
C.V. %	5.79	**Pred R-Squared**	0.7809
PRESS	7048.00	**Adeq Precision**	19.682

Fig.1: Parity plot showing the distribution of experimental vs. predicted values of protease production

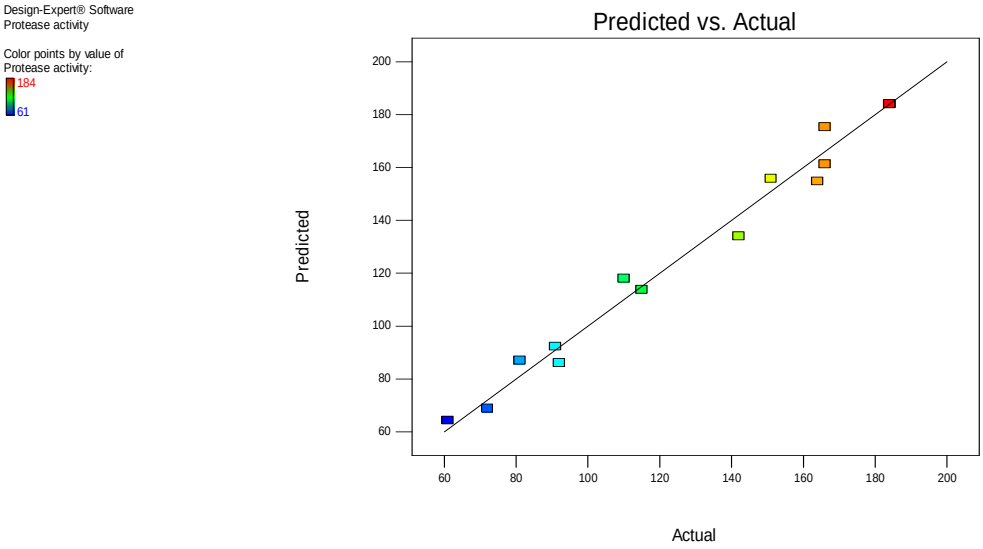


Fig. 2a: Contour plot showing the effect of optimized components on protease production

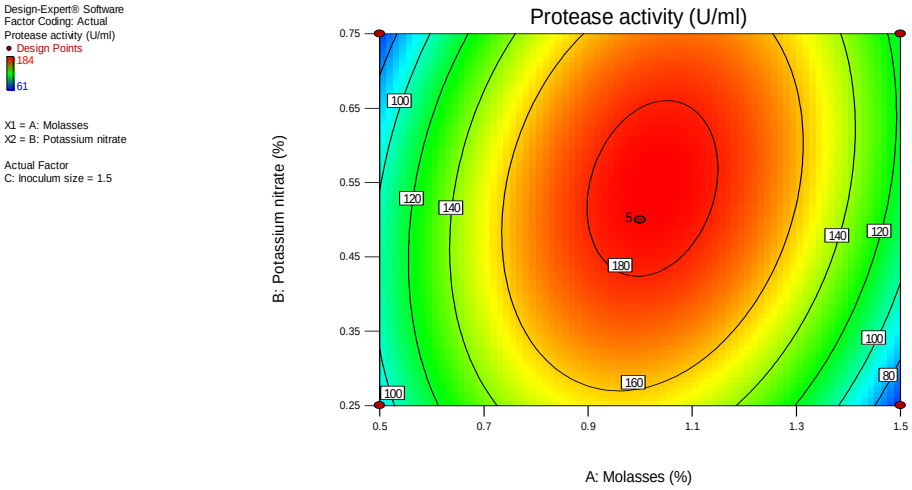


Fig.2b: Response plot showing the effect of optimized components on protease production

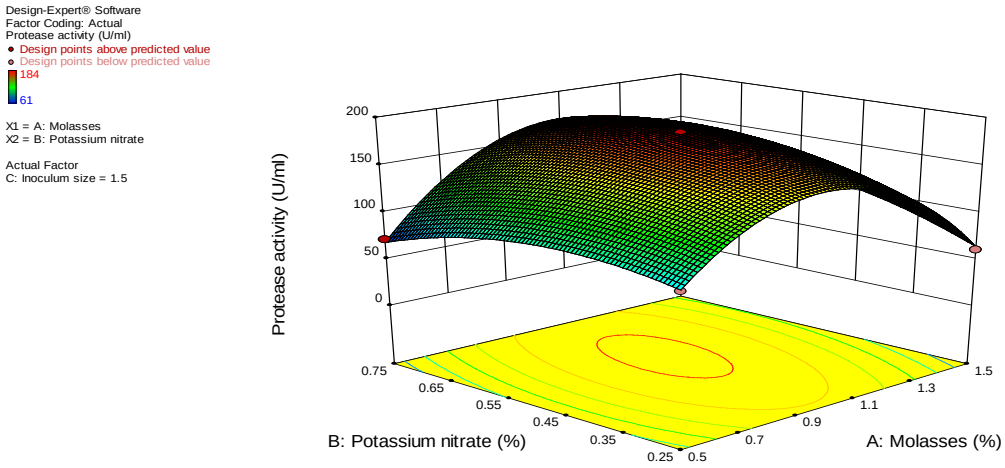
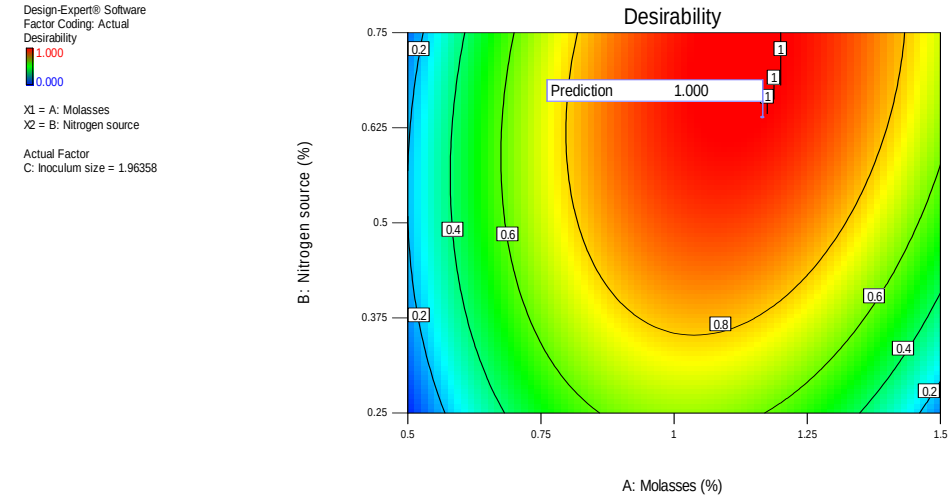


Fig. 3: Desirability for optimized media



CONCLUSION

The application of statistical design for screening and optimization of culture conditions allows quick identification of the important factors and interactions between them. The essential step in the use of statistical experimental design methods is to select the suitable ranges of the selected control factors in the initial experiments. In fact, most researchers arbitrarily adopt the range of tested values of each variable merely based on experience. Therefore, adequately choosing the initial test range would lead to the proper direction in approaching the optimum response. The eventual objective of RSM is to determine the optimum operating conditions for the system or to determine a region of the factor space in which operating specifications are satisfied. Since RSM is used to study the effects of several factors influencing the responses by varying them simultaneously in a limited number of experiments, it was thought to fit to the scope of this study. The CCD method allowed to study and explore culture conditions supporting changes in concentrations of medium components in just 17 experimental runs with an increase in protease production (205±0.35U/ml) over the basal medium(165±0.28 U/ml). This work has demonstrated the use of a central composite design by determining conditions leading to the maximum enzyme production. Central composite experimental design maximizes the amount of information that can be obtained, while limiting the numbers of individual experiments required. The enzyme activity predicted by the model at optimal conditions agreed fittingly with experimental data, thus confirming the model validity. Studies showed that this strain S8 has a good potential for further studies.

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